# Repair of Cisplatin−DNA Adducts by the Mammalian Excision Nuclease†

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ABSTRACT: Nucleotide excision repair is one of the many cellular defense mechanisms against the toxic effects of cisplatin. An in vitro excision repair assay employing mammalian cell-free extracts was used to determine that the 1,2-d(ApG) intrastrand cross-link, a prevalent cisplatin−DNA adduct, is excised by the excinuclease from a site-specifically modified oligonucleotide 156 base pairs in length. Repair of the minor interstrand d(G)/d(G) cross-link was not detected by using this system. Proteins containing the high mobility group (HMG) domain DNA-binding motif, in particular, rat HMG1 and a murine testis-specific HMG-domain protein, specifically inhibit excision repair of the intrastrand 1,2-d(GpG) and -d(ApG) cross-links. This effect was also exhibited by a single HMG domain from HMG1. Similar inhibition of repair of a site-specific 1,2-d(GpG) intrastrand cross-link by an HMG-domain protein also occurred in a reconstituted system containing highly purified repair factors. These results indicate that HMG-domain proteins can block excision repair of the major cisplatin−DNA adducts and suggest that such an activity could contribute to the unique sensitivity of certain tumors to the drug. The reconstituted excinuclease was more efficient at excising the 1,3-d(GpTpG) intrastrand adduct than either the 1,2-d(GpG) or d(ApG) intrastrand adducts, in agreement with previous experiments using whole cell extracts [Huang, J.-C., Zamble, D. B., Reardon, J. T., Lippard, S. J., Sancar, A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 10394−10398]. This result suggests that structural differences among the platinated DNA substrates, and not the presence of unidentified cellular factors, determine the relative excision repair rates of cisplatin−DNA intrastrand cross-links in the whole cell extracts.

The antineoplastic agent cisplatin [*cis*-diamminedichloroplatinum(II) or *cis*-DDP][1] has been one of the most successfully employed anticancer drugs following its approval for clinical use in 1979 (Loehrer & Einhorn, 1984). Although the mechanism of action is not completely understood, there is strong evidence to suggest that cisplatin exerts its cytotoxic effects by damaging DNA (Rosenberg, 1985). Support for this hypothesis is provided by the observation that DNA-repair-deficient cells are especially sensitive to the drug (Beck & Brubaker, 1973; Fraval et al., 1978; Dijt et al., 1988).

The major cisplatin−DNA adducts are 1,2-intrastrand d(GpG) and d(ApG) cross-links, which account for 90% of all the lesions (Fichtinger-Schepman et al., 1985) and form distinct structural elements (Takahara et al., 1995). In contrast to the minor adducts, which include various 1,3-d(GpNpG) intrastrand and DNA interstrand cross-links, the major 1,2-intrastrand adducts are not formed by the inactive isomer, *trans*-DDP, owing to geometric constraints (Lepre & Lippard, 1990). This difference has focused attention on the 1,2-intrastrand cross-links, as has the discovery of cellular proteins which specifically recognize these adducts and do not bind to DNA damaged by clinically ineffective platinum compounds (Chu, 1994). These proteins share a common structural element, the high mobility group (HMG) domain [for reviews, see Landsman and Bustin (1993), Grosschedl et al. (1994), and Baxevanis and Landsman (1995)]. HMG-domain proteins recognize and further bend DNA distorted by 1,2-intrastrand cisplatin−DNA adducts, but not the 1,3-intrastrand cross-links (Pil & Lippard, 1992; Chow et al., 1994). The mechanism by which this class of proteins might mediate cisplatin cytotoxicity has not yet been elucidated, although several models have been proposed (Donahue et al., 1990; Lippard, 1993). For example, specific binding of HMG-domain proteins to platinated DNA could shield the adducts from cellular defense mechanisms such as nucleotide excision repair, a hypothesis supported by studies in yeast (Brown et al., 1993; McA'Nulty & Lippard, 1996).

Repair of both the 1,2-d(GpG) (Huang et al., 1994) and 1,3-d(GpTpG) (Szymkowski et al., 1992; Huang et al., 1994) intrastrand cross-links by the human nucleotide excision repair pathway has been previously demonstrated. The 1,3-intrastrand adduct was a better substrate for the excinuclease, possibly because of specific shielding of the 1,2-intrastrand adduct by cellular factors such as HMG-domain proteins. In the present, complementary study, we have investigated this hypothesis by studying excision repair of the intrastrand adducts in a reconstituted system of highly purified repair components (Aboussekhra et al., 1995; Mu et al., 1995, 1996), thereby obviating any potential interference by other

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[1] Abbreviations: bp, base pair; CFE, cell-free extract; *cis*-DDP or cisplatin, *cis*-diamminedichloroplatinum(II); DMF, *N*,*N*-dimethyl formamide; FA, Fanconi's anemia; FPLC, fast protein liquid chromatography; HMG, high mobility group; HPLC, high pressure liquid chromatography; ICL, interstrand cross-link; nt, nucleotides; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; *trans*-DDP, *trans*-diamminedichloroplatinum(II); XP, xeroderma pigmentosum.

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factors present in cell extracts. In addition, we examined the ability of the excision nuclease to repair the second most abundant cisplatin−DNA adduct, the 1,2-d(ApG) intrastrand cross-link, as well as a minor adduct, the interstrand (dG)/(dG) cross-link. The ability of HMG-domain proteins and a single, isolated HMG domain to inhibit repair of various platinum−DNA adducts was also assessed both in whole cell extracts and with the reconstituted system. The results described provide a comparative analysis of excision repair of most cisplatin−DNA adduct types and present further evidence to support a role for HMG-domain proteins in modulating the anticancer activity of cisplatin.

## MATERIALS AND METHODS

*Materials.* Cisplatin was obtained as a gift from Johnson-Matthey. Recombinant rat HMG1 and HMG domain B (residues K86-K165 of HMG1) were expressed and purified as described (Pil & Lippard, 1992; Chow et al., 1995). Recombinant mouse testis-specific HMG protein was expressed and purified as described (Boissonneault & Lau, 1993; Ohndorf and Lippard, unpublished results). All proteins were stored in buffer A (50 mM Tris-HCl, pH 7.3, 50 mM NaCl, 5 mM 2-mercaptoethanol) at −20 °C. T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim. The [$\gamma$-$^{32}$P]ATP was purchased from Amersham or Dupont, NEN.

*Cell-Free Extracts (CFE).* The HeLa S3 cell line was from the stock of Lineberger Comprehensive Cancer Center (University of North Carolina) or was supplied by P. A. Sharp (MIT). The wild-type rodent cell line (AA8), the xeroderma pigmentosum complementation group F (XPF) rodent cell line (CHO-UV41), and the XPG rodent cell line (CHO-UV135) were obtained from the American Type Culture Collection Repository (Rockville, MD). Whole cell-free extracts (CFEs) from all cell lines were prepared by the method of Manley et al. (1980) and stored at −80 °C until use.

*Platinated Oligonucleotides.* Oligonucleotides were synthesized on a 1 $\mu$mol scale on a Cruachem PS250 DNA synthesizer and deprotected according to standard methods. All of the oligonucleotides to be platinated were converted to their sodium salts on a Dowex cation exchange resin (Aldrich) prior to platination.

*Site-Specific Intrastrand Adducts.* The synthesis of oligonucleotides containing the 1,2-d(GpG), the 1,2-d(ApG), and the 1,3-d(GpTpG) cisplatin intrastrand cross-links (Figure 1) was performed as described (Bellon et al., 1991).

*Site-Specific Interstrand Adduct.* The synthesis of the interstrand cross-link was performed in a manner analogous to that previously described (Brabec & Leng, 1993; Kasparkova & Brabec, 1995). Cisplatin was treated with 0.98 equiv of $AgNO_3$ for 14−16 h in DMF at room temperature in the dark, and the AgCl formed was removed by centrifugation. Platination was carried out with 0.4 mM DNA and a 4-fold molar excess of platinum to oligonucleotide strand in 10 mM $NaClO_4$ at 37 °C, and the reaction was stopped after 10 min by adding NaCl to 0.4 M. The monofunctional adduct (20-mer, ICL top strand, Figure 1a) was purified by ion exchange FPLC on a Mono Q HR5/5 column (Pharmacia Biotech) with linear gradients of 0.25−0.4 M NaCl in 20 mM Tris, pH 8.0, and then concentrated and washed with

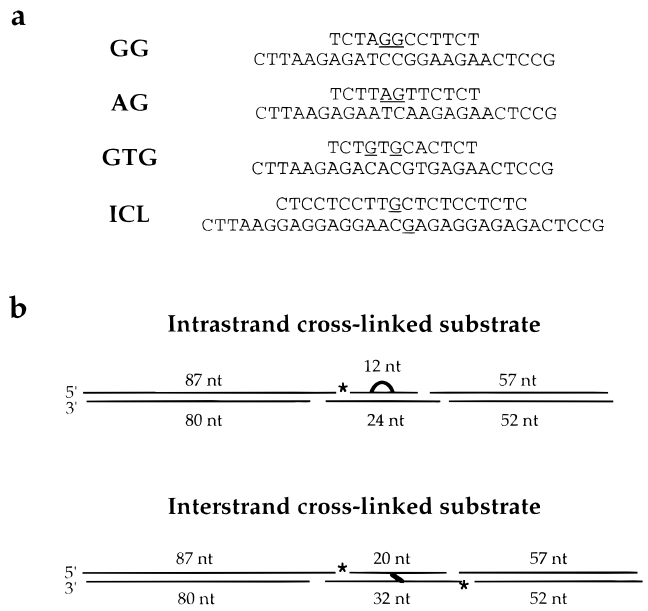


FIGURE 1: Platinated oligonucleotide constructs. (a) Sequences of oligonucleotides used to prepare site-specific cisplatin adducts. The complementary strands are indicated and the platinated nucleosides are underlined. (b) Schematic representation of full-length duplex substrates used in the in vitro excision repair assay. The sites of the radiolabels are marked by asterisks.

0.4 M NaCl at 4 °C in Centriplus-3 concentrators (Amicon). The site of platination was verified by Maxam−Gilbert sequencing analysis (Lemaire et al., 1991; Brabec & Leng, 1993). The platinated strand (20-mer) was allowed to anneal with 0.75 equiv of its complement (32-mer, ICL bottom strand, Figure 1a) in 0.45 M NaCl, 2 mM $MgCl_2$, 20 mM Tris-pH 7.4, for 2 h at room temperature and then for 2.5 h at 4 °C. The DNA was washed extensively with 100 mM $NaClO_4$, 1 mM $Mg(NO_3)_2$ in Microcon-3 concentrators (Amicon) at 4 °C and then incubated at 37 °C for 40 h. The interstrand cross-link was purified on a Mono Q HR5/5 column with linear gradients of 0.4−0.6 M NaCl in 10 mM NaOH. The DNA was concentrated in Centricon-3 concentrators (Amicon) at 4 °C, precipitated with ethanol, and stored in 5 mM Tris-pH 7.5, containing 50 mM $NaClO_4$. The presence of the interstrand cross-link was verified by 5′-end labeling of both the platinated and the unplatinated oligonucleotides with T4 polynucleotide kinase (NEB) and [$\gamma$-$^{32}$P]-ATP followed by analysis on 15% denaturing PAGE gels. The decreased mobility of the platinated oligonucleotide, which could be reversed by NaCN treatment (0.2 M, pH 10−11, 45 °C overnight), confirmed the presence of the cross-link. In addition, its location was determined by 5′-end labeling the monofunctionally platinated 20-mer or its complementary 32-mer prior to annealing the two strands. The interstrand cross-link was then isolated by 15% denaturing PAGE and analyzed by Maxam−Gilbert sequencing (Lemaire et al., 1991; Brabec & Leng, 1993).

*Cholesterol Substrate.* The cholesterol substrate was analogous to the cisplatin intrastrand cross-linked substrates depicted in Figure 1b. It was a 140 bp duplex with a cholesterol moiety attached to a propanediol backbone at position 70 of the damaged strand (Operon Biotechnologies, Almeda, CA). This cholesterol-containing duplex is an efficient substrate for the mammalian excinuclease and was prepared as previously described (Matsunaga et al., 1995).

*Intrastrand Cross-Linked Substrate.* Linear 156-bp duplexes were assembled from three sets of complementary oligonucleotides with overhangs 6 nt long (Figure 1b) following 5′ end-labeling of the damaged 12-mer with [$\gamma$-$^{32}$P]-ATP and T4 polynucleotide kinase (Huang et al., 1994). This procedure introduces a radioactive label at the fifth phosphodiester bond to the 5′ side of the site of damage for the GG and AG substrates and at the fourth phosphodiester bond to the 5′ side of the damage for the GTG substrate.

*Interstrand Cross-Linked Substrate.* The interstrand cross-linked substrate was also assembled from three sets of oligonucleotides (Figure 1b) except that both strands of the platinated duplex were 5′-end labeled with [$\gamma$-$^{32}$P]ATP before ligation to the annealed pairs of arms at 4 °C. The products were further purified after ligation by electrophoresis on 5% native polyacrylamide gels. The presence of the interstrand cross-link was verified by comparing the mobility of the substrate before and after NaCN treatment (0.2 M, pH 10−11, 45 °C overnight) on a 6% denaturing polyacrylamide gel. An impurity was detected, so the substrate was further purified by 6% denaturing PAGE. The product was not reannealed following this purification step, but digestion with restriction enzymes confirmed that the substrate contained double-stranded DNA (data not shown).

*Excision Assay with CFEs.* Whole cell extracts were used to measure excision of the damaged DNA according to a previously described method (Huang et al., 1994; Matsunaga et al., 1995). Briefly, all of the experiments were performed in a reaction buffer that contained 30 mM Hepes, pH 7.9, 40 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM ATP, 0.2 mM dithiothreitol (DTT), 20 $\mu$M of each dNTP, 0.2 mg/mL bovine serum albumin (BSA), 4.4 $\mu$g/mL pBR322 as carrier DNA, and 50−250 pM substrate. Several different types of assay conditions were used, but all reaction mixtures contained 100 $\mu$g of CFE and were incubated at 30 °C for 45 min unless otherwise indicated. For the complementation assay, a 50 $\mu$L reaction volume was used containing 20 $\mu$L of CFEs from XP complementation groups F or G, a mixture of the two, or HeLa in storage buffer (25 mM Hepes, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 16% glycerol). For the kinetic and protein inhibition studies, 25 $\mu$L reaction volumes were used, and 5 $\mu$L of CFE was added in storage buffer. The HMG-domain polypeptides were added in 5 $\mu$l of dilution buffer (50 mM Tris-pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol), and the reaction mixtures were incubated at 30 °C for 10 min prior to addition of CFE. The reactions were stopped by making them 0.5% SDS and 0.4 mg/mL proteinase K (Gibco BRL) and heated at 55 °C for 15 min. After two phenol/chloroform/isoamyl alcohol (25:24:1) extractions and an ether extraction, the DNA was precipitated with ethanol, washed with 70% ethanol, dissolved in formamide loading buffer [80% form amide (v/v), 10 mM EDTA, 1 mg/mL each xylene cyanol FF and bromophenol blue], and resolved by 10% denaturing PAGE. When the interstrand cross-linked substrate was used, reaction products were treated overnight with 0.2 M NaCN, pH 10−11, at 45 °C, and precipitated with ethanol prior to resolution on the gel. The gels were vacuum dried on Whatman 3 mm chromatography paper and exposed to X-ray film (Kodak, X-OMAT AR) at −80 °C with an intensifier screen. The level of excision was quantitated either by an AMBIS Systems scanner or by using a PhosphorImager with ImageQuant software (Molecular Dynamics).

*Reconstituted Excision Assay.* The excision assay with highly purified repair components was performed as previously outlined (Mu et al., 1996). The reaction buffer described above was used, except that the pBR322 and dNTPs were omitted and 0.4 M ATP (Boehringer Mannheim) was added separately to the reaction mixture. The purified repair factors were in 8 $\mu$l of storage buffer for a 25 $\mu$l reaction, and consisted of 20 ng of XPA, 1−2 ng of TFIIH, 7 ng of XPC, 1−2 ng of XPG, 1−2 ng of XPF/ERCC1 and 250 ng of RPA. All reaction mixtures were incubated at 30 °C for 2 h unless otherwise indicated. For the protein inhibition studies, HMG-domain polypeptides in 3 $\mu$L of buffer A were added, and the reaction mixtures were incubated for 10 min at 30 °C before the purified proteins were added.

*Mobility Shift Assay.* The intrastrand cross-linked probes were incubated in 20 $\mu$L of the excision assay reaction buffer containing 5 $\mu$L of tsHMG in dilution buffer for 30 min on ice, followed by the addition of 1 $\mu$L of gel loading solution (30% glycerol, bromophenol blue, and xylene cyanol FF) and electrophoresis on a prerun, precooled (4 °C), 0.5× TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3) 5% native polyacrylamide gel (29:1 acrylamide: *N*,*N*′-methylenebisacrylamide, 0.08% ammonium persulfate). Gels were vacuum dried and exposed to film (Kodak, X-OMAT AR) at −80 °C with an intensifier screen.

## RESULTS AND DISCUSSION

*Repair of the 1,2-d(ApG) Cisplatin Adduct in Human CFE.* To study repair of individual cisplatin−DNA intrastrand adducts, we constructed 156 bp site-specifically platinated substrates with a radiolabel located several bases to the 5′ side of the platinum lesion (Figure 1). Previously, we reported that the 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin−DNA cross-links are excised by the mammalian nucleotide excision repair pathway (Huang et al., 1994). As shown here, the second most abundant cisplatin−DNA lesion, the 1,2-d(ApG) intrastrand adduct, is similarly repaired by the human excinuclease (Figure 2). Incubation of the substrate with HeLa CFE produced a small ladder of bands, 25−29 nucleotides (nt) in length, indicating excision near the damaged site. Unlike the prokaryotic excinuclease system, the mammalian excision nuclease cutting pattern is variable, with variations occurring primarily at the 5′ incision, resulting in the observed ladder of bands (Svoboda et al., 1993; Mu et al., 1996). As shown in Figure 2, lanes 2 and 3, the ladder was not detected following incubation with CFEs from cell strains of xeroderma pigmentosum (XP) patients. XP is a condition arising from defective nucleotide excision repair (Cleaver & Kraemer, 1989). Defects in seven different genes of the nucleotide excision repair pathway give rise to seven complementation groups. When extracts from two different groups, in this case XPF and XPG, were mixed together and incubated with substrate, activity was restored and excision of the damaged DNA was observed (lane 4) (Reardon et al., 1993). Many types of DNA damage are repaired in this fashion, including a variety of adducts that distort the DNA helix (Sancar, 1994) such as the cyclobutane pyrimidine dimers produced by UV irradiation and the benzo[a]pyrene−guanine adducts caused by cigarette smoke. The work

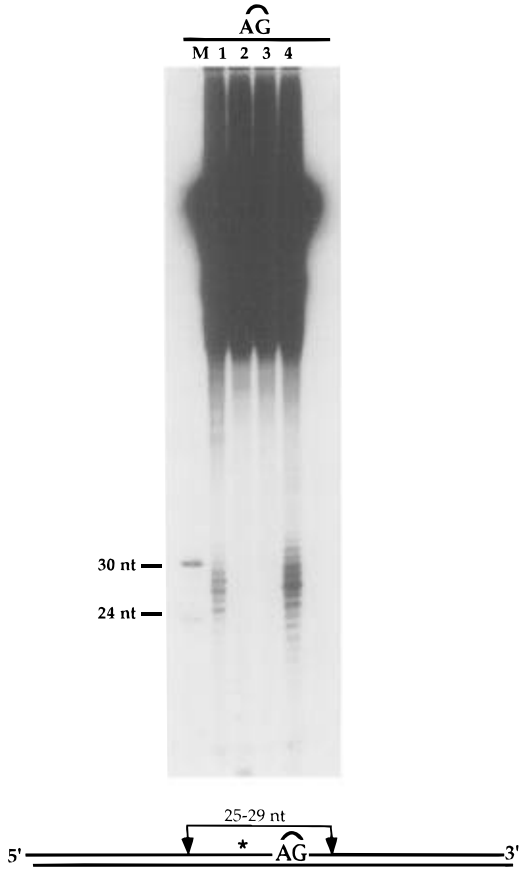


FIGURE 2: Excision repair of the 1,2-d(ApG) cisplatin adduct by the human excinuclease. The substrate was incubated with the CFEs of HeLa (lane 1), XPG (lane 2), XPF (lane 3), or a mixture of XPF and XPG (lane 4) for 45 min at 30 °C, and the excision products were analyzed on a 10% denaturing polyacrylamide gel. The bands corresponding to 25−29 nt located between the two markers are excision products, as indicated on the scheme at the bottom, which marks the incision sites of the human excinuclease. Lane M, 24 and 30 nt markers.

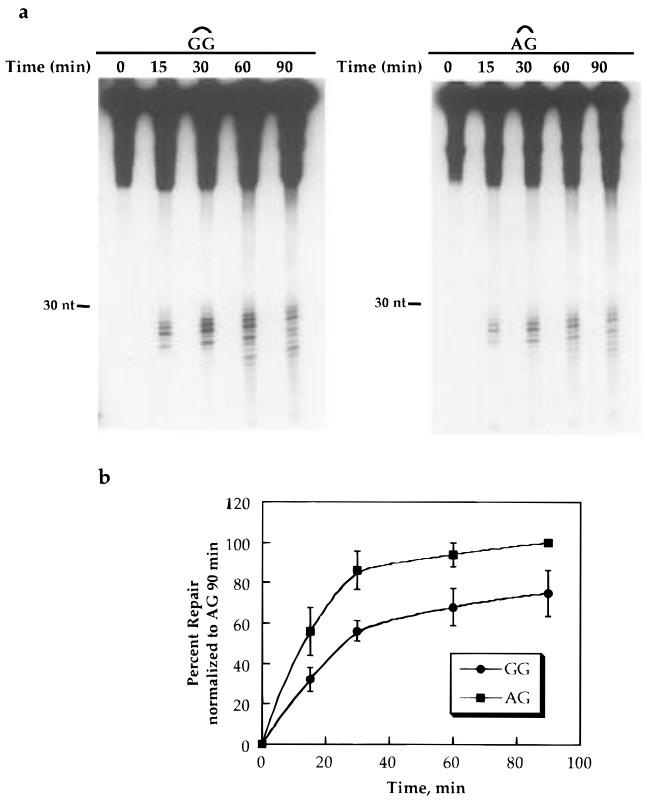


FIGURE 3: Kinetic studies of excision of 1,2-d(GpG) and 1,2-d(ApG) cisplatin intrastrand adducts by HeLa CFE. (a) Increasing amounts of excision products with time are revealed on denaturing 10% polyacrylamide gels. Substrates were incubated with the HeLa CFE for the times indicated. (b) Plot of three excision experiments such as that shown in panel a. The data for each experiment were normalized to the highest level of repair observed. Error bars represent ± 1 esd.

reported here, in conjunction with previous studies, demonstrates that the DNA distortions induced by the major cisplatin−DNA adducts are also recognized by the human excinuclease, resulting in excision of these lesions.

*Relative Rates of Removal of the Cisplatin 1,2-Intrastrand Adducts in Human CFE.* The relative rates of excision of the 1,2-intrastrand cross-links were determined by incubating the site-specifically platinated probes in HeLa CFE for increasing amounts of time (Figure 3). The 25−29 nt excision products were digested by nonspecific nucleases in the whole cell extract, resulting in the detection of additional bands corresponding to material smaller than 25 nt. The experiment shown in Figure 3 was repeated three times, but differences among preparations of CFEs caused some variability in the levels of excision. For this reason, data for each experiment were normalized to the highest level of repair (the 90 min time point for AG). The resulting plot reveals that both of the 1,2-intrastrand adducts are repaired in a similar fashion (Figure 3b). In all experiments, however, the 1,2-d(ApG) adduct was a better substrate for the excinuclease, with a level of repair about 30% higher than for the 1,2-d(GpG) adduct after 90 min of incubation.

Studies of prokaryotic excision repair reported the same relative rates of repair of the 1,2-intrastrand platinum−DNA adducts, although in this work the 1,2-diaminocyclohexane ligand was used in place of the ammine groups on platinum (Page et al., 1990). Both the d(GpG) and d(ApG) 1,2-intrastrand adducts bend and unwind the DNA to a similar degree (Bellon et al., 1991), so the small but reproducible difference in the relative rates of repair is not a result of global changes in DNA structure caused by platinum binding. The difference may result from local structural variations in the platinated bases, providing a slightly altered recognition element for the excinuclease. It should be noted that each adduct was studied in the context of a single sequence; sequence context effects on excision repair and other properties of cisplatin−DNA adducts are the subject of a separate investigation.

*Repair of the Interstrand Cross-Linked Substrate.* Cisplatin interstrand cross-links preferentially form between guanosine nucleosides on opposing strands in the 5′-d(GpC)-3′ sequence (Lemaire et al., 1991). These adducts result in dramatic conformational changes in DNA structure (Sip et al., 1992; Huang et al., 1995), and at least one adduct has been reported to be recognized specifically by HMG1 (Kasparkova & Brabec, 1995). Although interstrand cross-links comprise fewer than 5% of the lesions formed when platinum binds to DNA (Eastman, 1987), they have been implicated as a cytotoxic lesion (Roberts et al., 1987). Furthermore, although the cellular defenses against cisplatin are multifactorial (Scanlon et al., 1991; Chu, 1994), the processing of such interstrand adducts has been implicated as a mechanism of the cisplatin resistance of certain cell lines (Bedford et al., 1988; Zhen et al., 1992; Hill et al., 1994b).

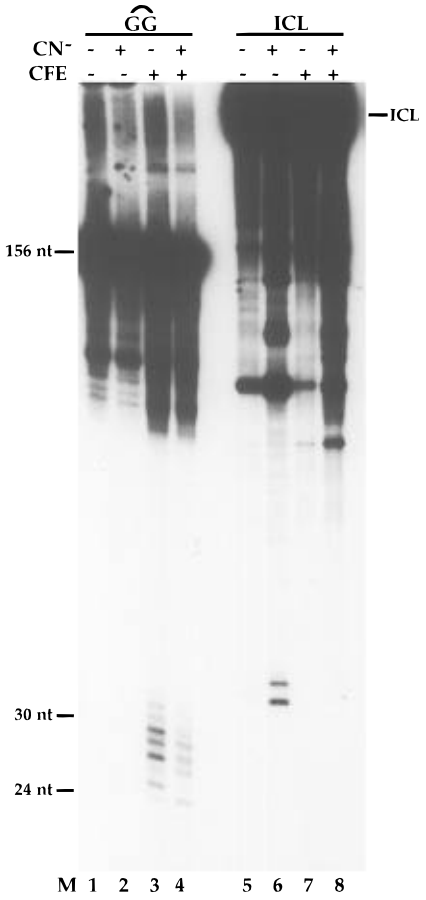


FIGURE 4: Excision of 1,2-d(GpG) intrastrand and interstrand cisplatin cross-links by mammalian excinuclease. The substrates were incubated with CHO AA8 CFE for one hour at 30 °C (lanes 3, 4, 7, and 8) and subsequently treated overnight with NaCN (lanes 2, 4, 6, and 8) prior to analysis on a denaturing 10% polyacrylamide gel. The 30 and 24 nt markers are indicated.

Thus, the possibility that interstrand cross-links contribute to the mechanism of action of cisplatin cannot be ruled out.

Accordingly, an excision repair substrate containing a site-specific cisplatin interstrand cross-link was prepared by the same method used for the intrastrand cross-linked substrates. In this case, however, the site-specific interstrand cross-link was constructed with two oligonucleotides, 20 and 32 nt in length (Figure 1a), and a radioactive label was introduced on both strands of the duplex insert (Figure 1b) in order to facilitate detection of cleavage sites on either strand. The interstrand cross-linked substrate was incubated under excision assay conditions and analyzed by denaturing PAGE (Figure 4). The gel was overloaded in order to facilitate detection of low levels of excision repair, resulting in the visualization of very small amounts of impurities surrounding the full length probes. Nevertheless, it is clear from the reduced mobility of the major band in comparison to the intrastrand cross-linked substrate that most of the material contains the interstrand cross-link.

In order to detect products arising from incision on only one strand of the duplex, the DNA was treated with NaCN after incubation with CFE using conditions which release the platinum adduct (Lemaire et al., 1991). The CHO AA8 CFE was employed for this experiment because it provides a high level of excision of the intrastrand cross-link. HeLa CFE afforded similar results (data not shown). The 1,2-d(GpG) intrastrand adduct was run as a positive control, to ensure that the extract was active in nucleotide excision repair (Figure 4, lane 3). After NaCN treatment, the pattern of bands resulting from excision of the control substrate increased in mobility by one nucleotide (compare lanes 3 and 4), as expected for removal of the positively charged platinum complex from the excised oligonucleotide fragments. Even after extensive native and denaturing polyacrylamide gel purification steps, however, NaCN treatment of the interstrand cross-linked substrate released two oligonucleotides having a slower mobility than that of the 30 nt marker (lane 6). The extra bands observed might arise from an interstrand cross-linked oligonucleotide ligated to the arms of the host DNA only at the ends of the 20-mer; incomplete NaCN reversal would result in the release of two products, an unmodified 32-mer and a platinated 32-mer. A recently reported solution structure determination of platinated DNA containing an interstrand cross-link revealed that this cisplatin adduct can cause marked disruption of the B−DNA duplex (Huang et al., 1995), which might account for the poor ligation efficiency observed in the present study. The two new bands were not observed when substrate was incubated with CFE prior to cyanide treatment (lane 8), presumably because of nuclease degradation. Of greater consequence, however, is that no excision products could be detected in the assay, either before or after NaCN treatment. Varying the incubation times and amount of substrate used did not alter this result (data not shown). Although a faint pattern of bands was observed in the range between the 30 and 24 nt markers, these features were no more prominent than in other regions of the autoradiograph and are ascribed to nonspecific nuclease activity.

Studies in cell strains derived from patients with Fanconi's anemia (FA) suggest that nucleotide excision repair may not be the primary pathway for removing the cisplatin interstrand cross-links (Plooy et al., 1985; Dijt et al., 1988). Although nucleotide excision repair can occur in FA cells, they are extremely sensitive to cross-linking agents (Fujiwara et al., 1977), and a deficiency in the repair of interstrand cross-links was cited as an explanation for their marked cisplatin sensitivity. DNA defects that have their own discrete repair pathways, such as mismatches, however, are also removed by nucleotide excision repair, albeit less efficiently (Sancar, 1994). Repair of the interstrand cross-link was detected by using a repair synthesis assay (Calsou et al., 1992). This type of assay measures the amount of new DNA synthesized after damage removal in whole cell extracts, however, so the signal could have been a result of repair other than nucleotide excision. In contrast, the excision assay can be used to distinguish between different types of repair.

The mammalian excinuclease hydrolyzes the phosphodiester bond located at a position five nucleotides to the 3′ site of the damage and 22−24 nucleotides to the 5′ site of the damage (Sancar, 1994). The interstrand cross-linked substrate used in this study contained a radioactive label at the 10th phosphodiester bond to the 5′ side of the damage on one strand of the duplex and at the 16th phosphodiester bond to the 5′ side of the damage on the other strand (Figure 1). Thus, if nucleotide excision occurred in the normal manner, it could be detected by this assay. The fact that we were unable to detect any nucleotide excision products of the cisplatin interstrand cross-linked substrate might be the result of impurities in our probe which somehow obscured its repair. We consider this possibility to be unlikely,

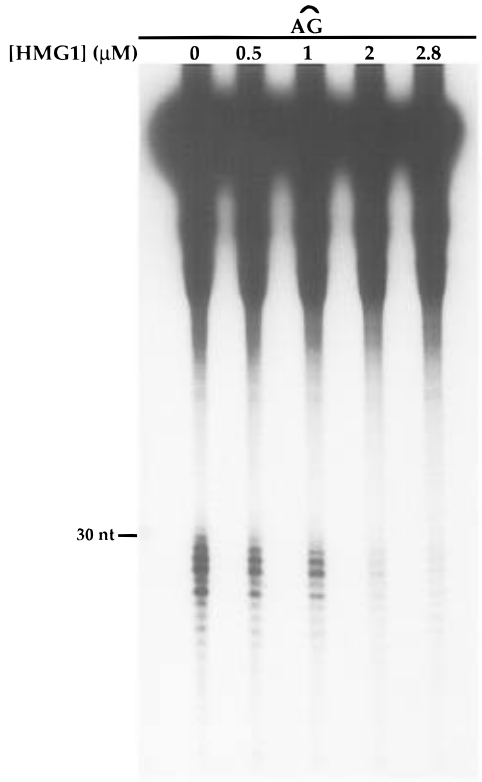


FIGURE 5: HMG1 inhibition of excision of the 1,2-d(ApG) cisplatin cross-link. The substrate was incubated with HMG1 at the concentrations indicated for 10 min at 30 °C followed by incubation for an additional 45 min in the HeLa CFE.

however, and conclude that, if the interstrand cross-link is removed by the mammalian nucleotide excision repair pathway, the efficiency is much lower than that of any of the intrastrand adducts studied by the excision assay.

*Inhibition of Excision Repair by HMG-Domain Polypeptides in HeLa CFE.* Of the HMG-domain proteins studied to date, almost all bind specifically to cisplatin-modified DNA (Brown et al., 1993; Chow et al., 1994; Treiber et al., 1994). In particular, HMG1 binds to both 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links but not to the 1,3-d(GpTpG) intrastrand adduct (Pil & Lippard, 1992). The effects of HMG1 on the excision of the 1,2-d(ApG) cisplatin intrastrand adduct are shown in Figure 5. Upon addition of the protein to the excision repair assay, the amount of excised products decreased with increasing concentrations of HMG1, and 2.8 $\mu$M concentrations of HMG1 resulted in a 70% drop in the amount of excision observed in HeLa CFE alone. Excision of the 1,2-d(GpG), but not the 1,3-d(GpTpG), adduct is also inhibited by the same concentrations of HMG1 (Huang et al., 1994). Moreover, repair of the 1,2-intrastrand cross-links is blocked by other members of the HMG-domain family (data not shown). Since the homology in this group of proteins is mainly in the DNA-binding domain (Landsman & Bustin, 1993), it is unlikely that this affect is a result of interactions between the HMG-domain proteins and the repair factors. In support of this hypothesis, excision of the major cisplatin intrastrand adducts was also inhibited by domain B of HMG1 (Figure 6 and data not shown). HMG1 domain B blocked the repair of the 1,2-d(ApG) probe, but the same concentrations of the polypeptide did not affect repair of a control substrate containing cholesterol, demonstrating that the shielding is specific for the cisplatin adduct.

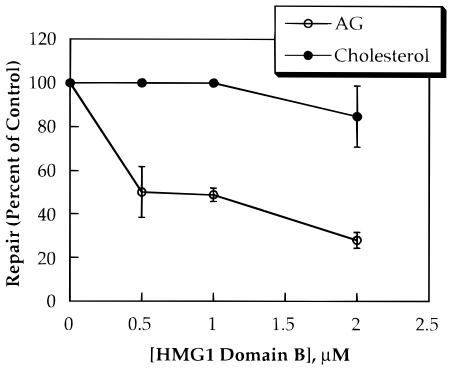


FIGURE 6: HMG1 domain B inhibition of excision repair of the 1,2-intrastrand cisplatin adducts. The 1,2-d(ApG) cross-linked substrate (open circles) and the cholesterol control substrate (closed circles) were incubated with HMG1 domain B at the concentrations indicated for 10 min at 30 °C prior to the addition of HeLa CFE and further incubation for 45 min. The level of repair without any HMG-domain protein was designated 100%. The cholesterol substrate was used here as a control for nonspecific binding of the polypeptide (see Materials and Methods). Data points are averages of two or three experiments, and the error bars represent ± 1 esd.

Analogous experiments were carried out with a testis-specific HMG-domain protein (tsHMG) (Boissonneault & Lau, 1993), since cisplatin is particularly effective in the treatment of testicular cancer (Loehrer & Einhorn, 1984). The binding specificity of this protein for cisplatin−DNA adducts was tested in an electrophoretic mobility shift assay (Figure 7a). Like HMG1, tsHMG binds specifically to the 1,2-d(GpG) intrastrand adduct, but not to the 1,3-d(GpTpG) cross-link. This difference is further manifest by the ability of tsHMG to inhibit repair of the 1,2-intrastrand but not the 1,3-intrastrand cross-link (Figure 7b). Furthermore, tsHMG specifically inhibits repair of the 1,2-d(GpG) intrastrand adduct at much lower concentrations than required for HMG1. At a concentration of tsHMG as low as 0.025 $\mu$M, 35% inhibition of excision of the 1,2-d(GpG) adduct was observed (Figure 7c), whereas 1−2 $\mu$M concentrations of HMG1 were necessary to achieve a similar level of inhibition (data not shown). Concentrations of tsHMG less than 0.05 $\mu$M actually stimulated the repair of the 1,3-d(GpTpG) adduct. At these concentrations of protein, only the 1,2-intrastrand adduct is specifically bound (Figure 7a), so repair of the cisplatin adducts is blocked by specific binding of the protein. Nonspecific binding inhibits repair at the higher concentrations of tsHMG. The fact that repair of the 1,2-d(GpG) adduct is blocked at concentrations of protein that do not inhibit repair of the 1,3-d(GpTpG) adduct suggests that this effect is not a consequence of nonspecific inhibition of the excinuclease by this protein.

The specific interaction of HMG-domain proteins with platinated DNA suggests that these proteins have the potential to mediate the mechanism of cisplatin cytotoxicity by one or more of several mechanisms (Donahue et al., 1990; Lippard, 1993). In an in vitro study, the 1,2-d(GpG) intrastrand adduct competed the HMG-domain protein hUBF away from its natural DNA-binding site (Treiber et al., 1994). This result supports a model in which the cisplatin−DNA adducts divert HMG-domain proteins from their normal cellular locus, with the potential to disrupt biological function. One series of experiments employing the yeast HMG-domain protein Ixr could not confirm this hypothesis in vivo, however (McA'Nulty et al., 1996). Two other models propose that the HMG-domain protein family influ-

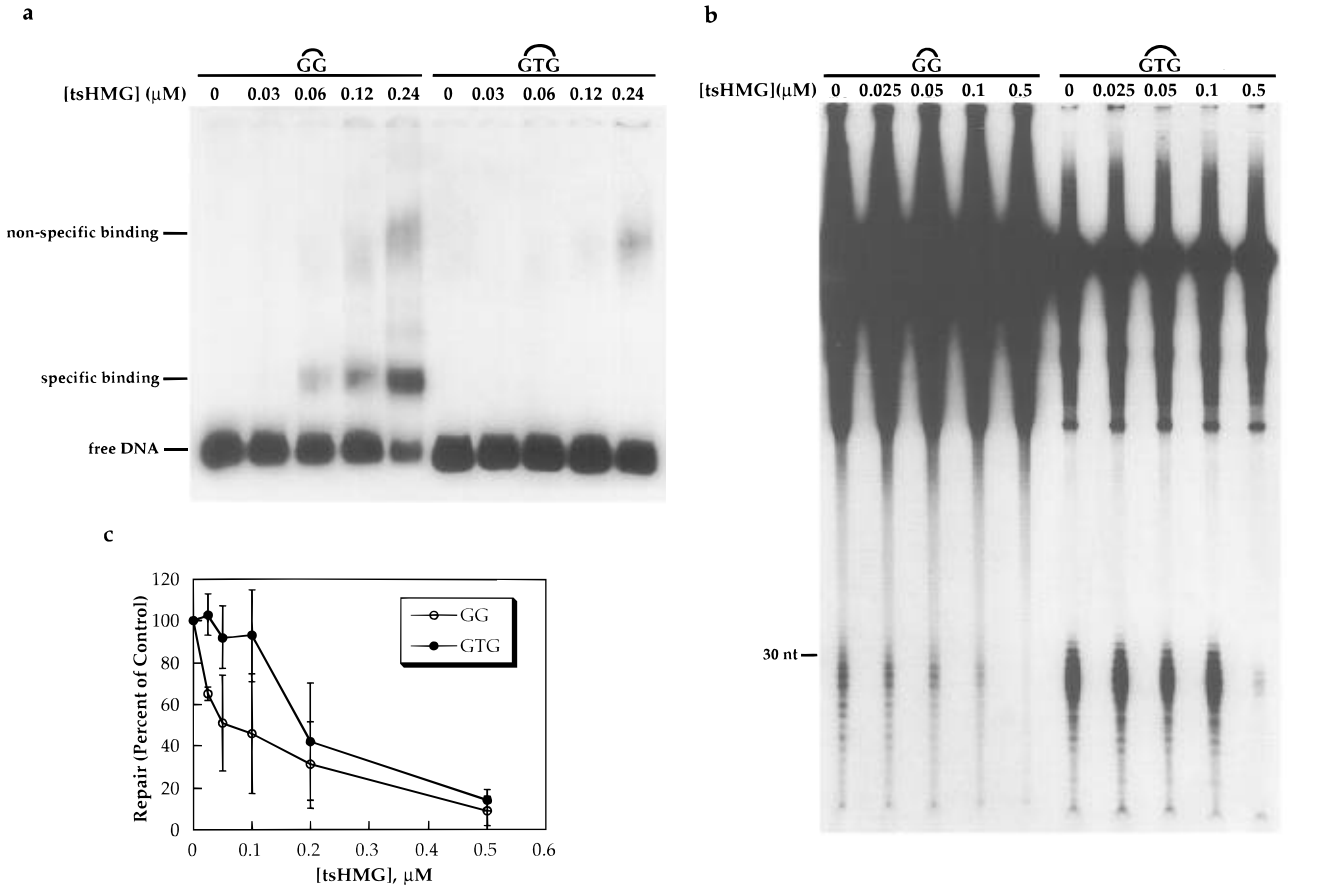


FIGURE 7: Effect of tsHMG on excision repair of the 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin−DNA adducts by the human excinuclease. (a) Mobility shift assay of the 1,2-d(GpG) and 1,3-d(GpTpG) intrastrand adducts. The substrates were incubated with the indicated concentrations of tsHMG for 30 min on ice under excision assay conditions prior to analysis on a native 5% polyacrylamide gel. (b) Denaturing 10% polyacrylamide gel showing inhibition of excision by tsHMG. (c) Plot of 5 excision experiments such as that shown in panel b. The 1,2-d(GpG) (open circles) and the 1,3-d(GpTpG) (closed circles) cross-linked substrates were incubated with tsHMG at the indicated concentrations for 10 min at 30 °C prior to addition of HeLa CFE. Several different preparations of HeLa CFE and tsHMG were used to compile the data, and data points are averages of four or five experiments. Error bars represent ± 1 esd.

ences the repair of cisplatin−DNA adducts. In the "damage recognition" model, HMG-domain proteins are postulated to assist excision of platinum damage by binding to the DNA adducts and serving as recognition elements for repair. The presence of HMG-domain proteins, however, does not stimulate repair of the 1,2-intrastrand cross-links, as we have demonstrated here and previously (Huang et al., 1994). A slight increase in excision of the 1,3-d(GpTpG) intrastrand cross-link was detected but appears to be a result of nonspecific binding.

Inhibition of the excinuclease by several different HMG-domain proteins as well as by an isolated HMG domain suggests that this protein class can modulate repair through a shielding mechanism. In this third model, binding of an HMG-domain protein to the platinum adducts prevents access of the repair complex to the site of damage. The support for the shielding model presented here follows on previous studies in yeast. Desensitization of *Saccharomyces cerevisiae* to cisplatin was achieved by interruption of the gene for the HMG-domain protein Ixr1 (Brown et al., 1993), a result which was subsequently linked to the excision repair pathway (McA'Nulty & Lippard, 1996). In another recent study an apparent deficiency in repair of 1,2-intrastrand cisplatin adducts in testicular teratoma cell lines was reported (Hill et al., 1994a). If tsHMG or a similar protein were expressed in testicular tumors, the significantly lower concentrations needed to achieve inhibition of repair of the major cisplatin adducts, as compared to other HMG-domain proteins, might explain the unique cisplatin sensitivity of this tumor type.

*Repair of the Cisplatin Intrastrand Adducts in a Reconstituted System.* There are many cellular proteins that bind to cisplatin−DNA adducts and might, in this manner, affect their processing. Proteins such as HMG1 specifically recognize 1,2-intrastrand adducts and could selectively block access of the excinuclease needed to repair these lesion. In contrast, the 1,3-intrastrand cross-links are poor targets for recognition by HMG1 and its analogues. In order to eliminate the possibility that HMG-domain proteins or some other protein class might interfere with excision repair of site-specifically platinated DNA, we compared the relative rates of repair in a reconstituted system of highly purified components (Mu et al., 1996) (Figure 8). The repair signal of all three intrastrand adducts increased with time over a 3 h period. This result differs from excision in the whole cell extracts where repair plateaued after 30−40 min. Only a small amount of repair (0.5−2%) was observed in the reconstituted system. Previous work demonstrated that the repair efficiencies of cyclobutane pyrimidine dimer and cholesterol adducts were 10% and 1%, respectively, although there was large variability in the levels of repair observed, as in CFE (Mu et al., 1996). In contrast to experiments performed with extracts, however, higher levels of repair were achieved with longer incubation times.

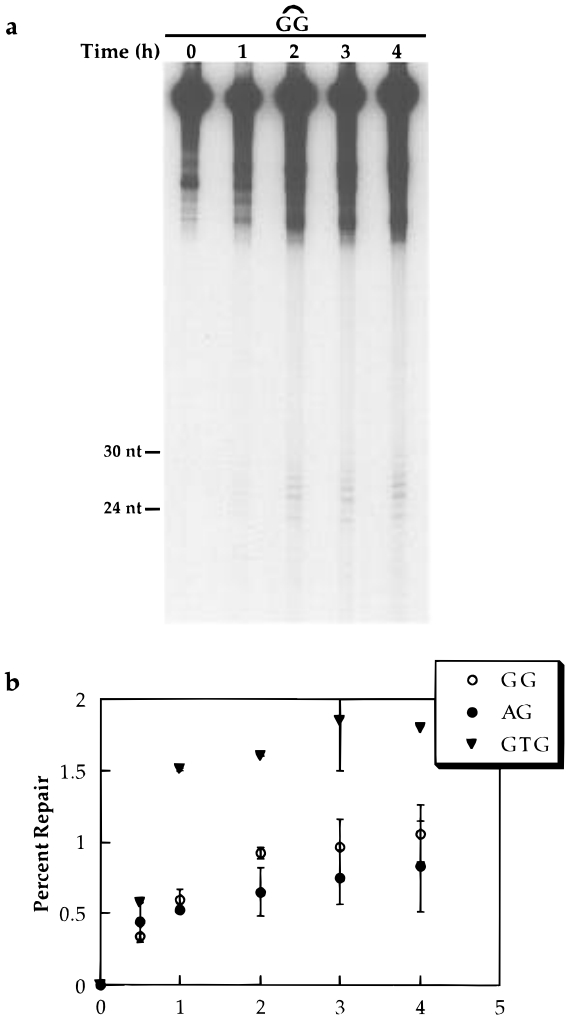


FIGURE 8: Kinetics of excision of cisplatin-intrastrand adducts by reconstituted excision repair. (a) The 1,2-d(GpG) intrastrand cross-linked substrate was incubated with the purified repair factors at 30 °C for the times indicated. Increasing amounts of excision products with time are revealed on a denaturing 10% polyacrylamide gel. (b) Plot of excision experiments of the 1,2-d(GpG) (open circles), 1,2-d(ApG) (closed circles), and 1,3-d(GpTpG) (triangles) cross-linked substrates, such as that shown in panel a. Each data point is an average of two or three experiments, the error bars representing ± 1 esd.

In the reconstituted system, the 1,3-d(GpTpG) adduct is excised most rapidly. This finding agrees with that in a previous study in cell-free extracts which demonstrated that the 1,3-intrastrand adduct is a better substrate for the excinuclease than the 1,2-d(GpG) cross-link (Huang et al., 1994). Thus, there appear to be no unidentified factors in the CFE which differentially interfere with repair of cisplatin−DNA adducts. The 1,3-d(GpTpG) intrastrand cross-link unwinds DNA by 23°, nearly twice the value of 13° observed for the 1,2-intrastrand adducts (Bellon et al., 1991). The more efficient repair of the 1,3- compared to the 1,2-intrastrand cross-links even in the reconstituted repair system suggests that structural differences in platinated DNA and not the presence of extraneous cellular proteins are responsible for the faster repair rate. An examination of the rate of incision of cisplatin−DNA adducts by the bacterial UvrABC endonuclease demonstrated the 1,3-d(GpCpG) intrastrand adduct to be incised less efficiently than the 1,2-d(GpG) intrastrand cross-link (Visse et al., 1994). The rate of UvrC binding to the 1,3-d(GpCpG) preincision complex

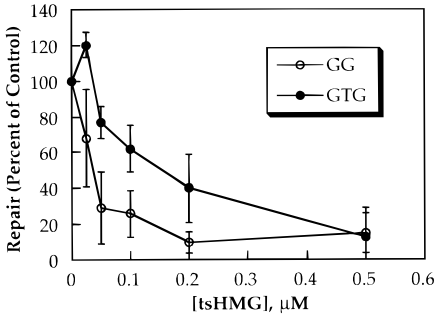


FIGURE 9: tsHMG inhibition of reconstituted excision repair of the 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin−DNA adducts. The 1,2-d(GpG) (open circles) or 1,3-d(GpTpG) (closed circles) cross-linked substrates were incubated with tsHMG at the concentrations indicated for 10 min at 30 °C prior to the addition of the purified repair proteins and further incubation for 110 min. Each data point is an average of three experiments, the error bars representing ± 1 esd.

was faster than to the 1,2-d(GpG) preincision complex, however. Thus both mammalian and prokaryotic excision repair systems can distinguish between the two types of cisplatin−DNA intrastrand cross-links, but differences between the pathways are reflected in the relative rates of repair of the adducts.

The relative rates of excision of the two 1,2-intrastrand adducts differed in whole cell extracts compared to the reconstituted system. In the HeLa extracts, the 1,2-d(ApG) adduct was reproducibly excised more efficiently than the 1,2-d(GpG) adduct. With the reconstituted excinuclease, however, over a period of several hours the rates of repair were indistinguishable (Figure 8b). This result demonstrates that the slightly faster rate of excision of the 1,2-d(ApG) adduct observed in the whole cell extracts is not caused by differential recognition of the two adducts by the excinuclease. Instead, the possibility of a cellular factor which can distinguish between the 1,2-intrastrand adducts and affect the relative rates of repair seems likely. Recently, the 1,2-d(ApG) cross-link was postulated to be a candidate for the cytotoxic lesion of cisplatin, based on platinum−DNA adduct levels (Fichtinger-Schepman et al., 1995). This result might be explained by a cellular component that contributes to differential processing of the two adducts. Since the 1,2-d(ApG) cross-link was repaired at least as efficiently as the other 1,2-intrastrand adduct, however, any difference in cytotoxicity between the two 1,2-intrastrand adducts can not be ascribed to excision repair.

*Inhibition of Excision Repair by tsHMG in a Reconstituted System.* Inhibition of repair of the cisplatin−DNA adducts by tsHMG was also tested in the reconstituted repair system. We chose to study tsHMG in this system rather than other HMG-domain proteins because it specifically blocks excision repair at lower concentrations than all the other proteins examined to date. As in whole cell extracts, at concentrations of tsHMG as low as 0.025 $\mu$M the excision of the 1,2-d(GpG) intrastrand adduct was inhibited while the repair of the 1,3-d(GpTpG) intrastrand adduct was slightly stimulated (Figure 9).

HMG-domain proteins interact with other cellular factors to regulate transcription and cellular differentiation and can facilitate the formation of larger nucleoprotein complexes (Grosschedl et al., 1994). Interactions with other proteins such as TBP (Ge & Roeder, 1994; Stelzer et al., 1994;

Shykind et al., 1995), the progesterone receptor (Onate et al., 1994), the transcription factors Oct1 and Oct2 (Zwilling et al., 1995), RNA polymerase I (Schnapp et al., 1994), and c-myc (Bunker & Kingston, 1995) have been reported. Our experiments with the reconstituted system address the possibility that inhibition of repair of cisplatin adducts is affected by HMG-domain proteins interacting with other factors in the whole cell extract. Specific inhibition of repair of the 1,2-d(GpG) intrastrand adduct is observed by using the same range of concentrations of tsHMG in both HeLa and in the reconstituted system. This result suggests indirectly that the inhibition of repair is not regulated, either positively or negatively, by interactions between the HMG-domain protein and any other cellular constituents. The tsHMG was isolated from mouse, but the CFE used was from a human cell line; so further examination of this possibility with an HMG-domain protein obtained from the same cell line used to make the extract would be of interest.

HMG-domain proteins are ubiquitous and present in HeLa extracts as indicated by western and southwestern blots (Toney et al., 1989; data not shown), but the relative rates of repair of the intrastrand DNA adducts were similar to those observed in the reconstituted system. This result suggests that the detectable levels of HMG-domain proteins in the extracts were not sufficient to block repair of the 1,2-intrastrand adducts. Inhibition of the mammalian excinuclease by adding HMG-domain proteins in this in vitro assay, however, supports the model in which these proteins shield cisplatin−DNA adducts from repair, thus sensitizing the cells to the drug. Overexpression of any protein that specifically recognizes cisplatin-modified DNA should thus enhance the toxicity of cisplatin and may be the cause of the unusual sensitivity of certain tissues to the drug.

## CONCLUSIONS

It has now been demonstrated that the three major cisplatin−DNA adducts, the 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpTpG) intrastrand cross-links, are repaired by the mammalian excinuclease. In a reconstituted system of highly purified proteins, the 1,3-intrastrand adduct is the most efficiently repaired, and the relative repair rates of the two 1,2-intrastrand adducts are very similar, although in whole cell extracts the 1,2-d(ApG) cisplatin−DNA adduct is excised at a slightly faster rate than the 1,2-d(GpG) cross-link. Repair of the 1,2-intrastrand adducts in the in vitro excision repair assay is specifically blocked by HMG1, an HMG1 DNA-binding domain, domain B, and by other HMG-domain proteins. Excision repair of the minor interstrand cross-link has not been detected by the excision repair assay.

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